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16. ABSTRACT

Several environmental contaminants, notably dibromochloropropane (Whorton et al. 1977) and kepone (Taylor et al. 1978; Cannon et al. 1978) have been implicated in sperm deficiencies among occupationally exposed males. These incidents emphasize the need for adequate testing of chemicals for effects on the male reproductive system. Although important in clinical diagnosis, the evaluation of sperm motility has not been used extensively as a tool in chemical toxicology, particularly in commonly used small laboratory species. Measurements of sperm motility parameters are complicated by several variables including sample manipulation, temperature, cell concentration, time factors and the method of quantitation. In the present study, a simple objective procedure to estimate the proportion of motile epididymal spermatozoa in the rat has been developed. Emphasis is given to details of handling the sperm sample to minimize intersample variation due to external factors. Quantitation of progressively motile spermatozoa is achieved simply by standard manual and electronic blood cell counting techniques. The difficulty in visual tracking of multiple motile spermatozoa is circumvented by counting only nonmotile cells. An index of motility is calculated from the difference in the nonmotile and total counts, each made on separate aliquots of a sperm suspension. The procedure described is an elaboration of a technique used by Mason and Thompson, 1977. The modified technique is demonstrated in rats given selected doses of dinoseb (2-sec-butyl 4,6-dinitrophenol), a testicular toxicant in the rat (Linder et al. 1982) and α-chlorohydrin (3-chloro-1,2-propanediol) whose antimotility effects have been documented in several species (Jones 1983).

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Measurement of Epididymal Sperm Motility as a Test Variable in the Rat

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Several environmental contaminants, notably dibromochloropropane (Whorton et al. 1977) and kepone (Taylor et al. 1978; Cannon et al. 1978) have been implicated in sperm deficiencies among occupationally exposed males. These incidents emphasize the need for adequate testing of chemicals for effects on the male reproductive system. Although important in clinical diagnosis, the evaluation of sperm motility has not been used extensively as a tool in chemical toxicology, particularly in commonly used small laboratory species. Measurements of sperm motility parameters are complicated by several variables including sample manipulation, temperature, cell concentration, time factors and the method of quantitation. In the present study, a simple objective procedure to estimate the proportion of motile epididymal spermatozoa in the rat has been developed. Emphasis is given to details of handling the sperm sample to minimize intersample variation due to external factors. Quantitation of progressively motile spermatozoa is achieved simply by standard manual and electronic blood cell counting techniques. The difficulty in visual tracking of multiple motile spermatozoa is circumvented by counting only nonmotile cells. An index of motility is calculated from the difference in the nonmotile and total counts, each made on separate aliquots of a sperm suspension. The procedure described is an elaboration of a technique used by Mason and Thompson, 1977. The modified technique is demonstrated in rats given selected doses of dinoseb (2-sec-butyl 4,6-dinitrophenol), a testicular toxicant in the rat (Linder et al. 1982) and α-chlorohydrin (3-chloro-1,2-propanediol) whose antimotility effects have been documented in several species (Jones 1983).

MATERIALS AND METHODS

Measurements were made in 110–140 day-old male Sprague-Dawley rats (Charles River Breeding Farms, Inc.). Sperm samples were diluted in a medium consisting of M199-Hank's salts (GIBCO™) fortified with an antibiotic mixture, PSN100X (GIBCO™) and bovine serum albumin (No. A-4378, Sigma Chemical Co.) in concentrations of 1% and 0.5%, respectively.

The rats were anesthetized with ether and killed by severing the abdominal aorta. The cauda epididymis was excised and the fundus

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of the vas deferens grasped with a curved hemostat. Adherent tissue was trimmed from the cauda and the surface cleansed with saline. A slight pressure was applied with the hemostat and a shallow cut (1-2 layers of coils) was made with iris scissors at a point where the engorged epididymal coils were visibly prominent. The extruded fluid was aspirated into a weighed 10-microliter capillary tube (Volupette™) which had been marked at 12.5 mm (approximately 2.5 mg of fluid) and the fluid sample was weighed. The capillary tube was inserted into a short piece of silicon tubing affixed to a syringe containing medium, and the sperm sample was flushed into a 5-ml vial containing approximately 3 ml of medium. The vial was filled with medium to form a convex meniscus, then capped with a teflon-faced silicon-septum screw cap (Wheaton Scientific) so that no air bubbles were visible when the vial was inverted. Excluding air bubbles from the suspension allowed the use of a vortex mixer to achieve rapid uniform distribution of spermatozoa without apparent trauma to the cells. This step was taken after preliminary work indicated that even relatively gentle inversion mixing of partially filled vials could decrease the number of motile cells.

After gentle inversion to disperse the sperm clot, the sample was agitated on a vortex mixer for 5 sec. An aliquot was immediately withdrawn and both chambers of an improved Neubauer hemacytometer (American Optical) were filled and immediately examined for nonmotile spermatozoa. The heads of all sperm cells which did not exhibit progressive motility (twitching cells were considered nonmotile) that were within or touching the outer perimeter grid lines of the central square millimeter were counted. A small incubator and a thermostatically controlled dry bath were used to maintain the temperature of the medium, sperm suspension and hemacytometer at approximately 37°C until the nonmotile count was initiated. A total sperm count was made on a second aliquot of the sperm suspension using an electronic particle counter (Coulter Counter™, Model ZF). The count was made on a 1:100 dilution of the sperm suspension in Isoton™ using a draw volume of 0.5 ml and a 100μ aperture tube. Instrument settings were 1, .707, and 10 for 1/aperture, 1/amplification, and lower threshold, respectively. Prior to each use the instrument reading was referenced with commercial blood standards (4C™, Coulter Diagnostics). For the total counts the sperm suspension was thoroughly mixed by inversion on a blood mixer (vortexing of partially filled vials caused foaming and erratic counts) just prior to making the final dilution in Isoton™.

An index of motility (MI) was calculated as:

$$MI = 1 - \left| \frac{H_1 + H_2}{0.04E} \right| \times 100$$

where H_1 and H_2 are the nonmotile counts for each hemacytometer chamber and 0.04 E is the average of 3 electronic counts (E) for total spermatozoa multiplied by the dilution factors reduced to simplest terms. Of course the total as well as nonmotile counts can also be made in the hemacytometer after killing the spermatozoa

by freezing, vortexing with air, or adding a spermicide [MI = 1-(nonmotile count ÷ total count) x 100]. The total counts can be made at any convenient time since the samples can be frozen indefinitely. The MI is an approximation of percent motility, however, hemacytometric and electronic counts will not correspond exactly and, in samples with poor motility, the expected variation in counts may yield negative MI values.

Several sources of variability were investigated. Since the method assumes equivalent distribution of spermatozoa in aliquots taken for nonmotile and total counts, the adequacy of distribution was determined. Ten samples of epididymal fluid were collected and diluted as described and electronic counts were made after 5 sec of vortex mixing. To estimate the uniformity of cell distribution, 2 subsequent aliquots from each sample were counted again after further mixing of the sperm suspensions by inversion.

To evaluate the reproducibility of hemacytometric and electronic sperm counts, epididymal fluid was collected and dispersed (1 mg/ml) into medium and additional dilutions of 0.75, 0.50, 0.25, and 0.125 mg/ml were prepared. Each concentration was subdivided into 10 aliquots of 2 ml each and hemacytometric and electronic counts made on each aliquot.

The effect of spermatozoa concentration on motility values was also evaluated. From each cauda of 5 rats, epididymal fluid samples of approximately 5, 3.75, 2.5, and 1.25 mg were collected and dispersed into 5 ml of medium. The MI was determined as described and the study was performed in replicate.

To assess the effect of incubation time on motility values, samples of epididymal fluid were collected from groups of 5 rats each and nonmotile sperm counts made after 0 (10-20 minutes post mortem) 1, 2, 2.5, 3, 3.5, and 4 hr of incubation at 37°C, and the MI was determined.

Contaminating nongerminal cells in the central square millimeter of the hemacytometer chambers were counted in epididymal fluid samples from the left and right caudae of 70 control animals for which motility was evaluated by the described procedure.

The technique was tested in rats treated with dinoseb or α-chlorohydrin. Groups of 10 male 97 day-old rats were fed diets containning 0, 125, 150, or 175 ppm of dinoseb. All rats were sacrificed on day 25 of treatment. α-Chlorohydrin was administered i.v. (tail vein) to groups of 10 male 120 day-old rats. Various concentrations of the compound were dissolved in normal saline and given at the rate of 2.5 ml/kg body weight in a single injection to obtain dosages of 0, 300, 375, and 450 mg/kg. The animals were sacrificed 3 hr after the injection and the MI was determined. Motility data in the various treatment groups were evaluated by analysis of covariance (SAS Users Guide, 1982) using the nonmotile and total spermatozoa per mg of epididymal fluid as the dependent and independent

variables, respectively. Probabilities for differences in the least square means were calculated and the significance level taken as $p < 0.05$.

RESULTS AND DISCUSSION

The MI and sperm cell concentrations for control animals from 11 miscellaneous data sets are given in Table 1. The mean MI (left and right caudae combined) for individual data sets ranged from 54.1 - 67.7. The mean of all samples combined was 61.3 (S.E. ± 0.9) with a coefficient of variation of 17.3%. Values for the left and right caudae were similar.

In the sperm suspensions evaluated for uniform distribution of spermatozoa, adequate dispersion of cells was indicated. Electronic counts made after 5 sec of vortex mixing and counts made in 2 subsequent repetitions were similar. The means of the 3 series of counts were 5753 ± 135, 5719 ± 105, and 5735 ± 132.

Table 1. Sperm Motility in Control Animals[a]

Data Set	N[b]	Motility Index			Sperm/ml
		Left	Right	Combined[c]	x 10^6
1	10	66.9 ± 10.4	56.9 ± 13.2	61.9 ± 12.6	1.27 ± 0.06
2	6	52.5 ± 7.0	60.5 ± 10.5	56.5 ± 9.5	1.15 ± 0.13
3	5	60.4 ± 5.5	58.8 ± 3.1	59.6 ± 4.3	1.29 ± 0.08
4	5	57.8 ± 13.6	61.1 ± 4.1	59.5 ± 9.6	1.34 ± 0.08
5	5	53.7 ± 15.9	54.6 ± 12.3	54.1 ± 13.5	1.23 ± 0.22
6	5	67.0 ± 3.4	68.4 ± 4.0	67.7 ± 3.6	1.37 ± 0.22
7	5	58.7 ± 9.0	64.1 ± 8.1	61.4 ± 8.6	1.25 ± 0.08
8	5	57.5 ± 16.6	59.7 ± 14.4	58.6 ± 14.7	1.24 ± 0.12
9	10	68.7 ± 7.4	61.3 ± 11.1	65.0 ± 9.9	1.26 ± 0.07
10	5	64.0 ± 5.4	61.4 ± 4.1	62.7 ± 4.7	1.32 ± 0.04
11	10	65.1 ± 12.9	60.8 ± 11.6	63.0 ± 12.1	1.26 ± 0.06
Total	71	62.2 ± 11.2	60.5 ± 10.1	61.3 ± 10.6	1.27 ± 0.11

[a] Values are mean ± SD
[b] N = Caudae
[c] 2N

The precision of the hemacytometric and electronic sperm counts was evaluated in 5 concentrations of epididymal fluid (0.125, 0.25, 0.50, 0.75 and 1 mg/ml). Both manual and electronic counts were linear over the concentrations measured and highly correlated (r = 0.99). The mean coefficients of variation were 12.9 and 3% for hemacytometric and electronic counts, respectively. Variation was greatest at the most dilute sperm cell concentration.

The use of the electronic counter reduced technician fatigue and enhanced the precision of the total sperm count. The error in the electronic counts due to contaminating nongerminal cells was estimated from hemacytometric counts of extraneous cells. Visual counts of contaminating cells in the hemacytometer central square indicated 0-6 cells/0.1 mm^3. The overall average was 1.4 cells/0.1 mm^3 or 14,000 contaminating cells/ml of sperm suspension. Based on the mean sperm cell concentration in Table 1, the approximate error in the electronic counts attributable to nongerminal cells was 1.1 %. No correction factor was deemed necessary.

Sperm motility values can be affected by dilution of spermatozoa in vitro (Bishop 1962). The effect of dilution on MI under conditions described in the present study is presented in Table 2. We chose approximately 0.5 mg of epididymal fluid/ml of medium as a compromise between a dilution which gave maximum motility values and one which offered ease in visual counting of nonmotile spermatozoa. Approximately 2.5 mg of epididymal fluid suspended in 5 ml of medium gave an average concentration of 1.27×10^6 spermatozoa/ml (Table 1). A twofold increase in sperm concentration (Table 2) did not appreciably increase the MI, but the difficulty in visual discrimination of nonmotile cells was greater. On the other hand, decreasing

Table 2. Effect of Spermatozoa Concentration on Motility

Data Set[a]	Sample Weight mg	mg/ml	Spermatozoa/ml[b] 10^6	Motility Index[b]
Replicate 1	5.2	1.04	2.89 ± 0.10	69.7 ± 1.1[c]
	3.8	0.76	2.02 ± 0.06	66.4 ± 2.0[c]
	2.4	0.48	1.36 ± 0.06	67.3 ± 1.1[c]
	1.3	0.26	0.68 ± 0.03	60.3 ± 2.3
Replicate 2	5.1	1.02	2.51 ± 0.08	65.3 ± 1.7[c]
	3.8	0.76	1.79 ± 0.05	62.1 ± 3.2
	2.5	0.50	1.25 ± 0.02	61.4 ± 2.7
	1.2	0.24	0.64 ± 0.02	56.4 ± 3.9

[a] Four samples taken from each cauda of 5 rats for each replicate
[b] Values are mean ± SE (N=10 caudae)
[c] Differs from motility values of lowest sperm concentration $p < 0.05$

the sperm cell concentration by 50% did not appreciably increase the ease of counting, but there was some evidence of decreased numbers of motile spermatozoa.

In practice, the motility values were based on nonmotile sperm counts made as quickly as possible after killing the animal, usually within 10-20 minutes. No significant change in the number of motile spermatozoa was apparent up to 4 hr postmortem when the sperm suspensions were incubated at 37°C (Table 3). Minor fluctuations in sample-to-sample incubation time therefore had negligible effects on motility values.

Table 3. Effect of Incubation Time on Sperm Motility[a]

Incubation Time (Hours)	Motility Index
0	62.1 ± 4.8
1	62.3 ± 2.3
2	58.8 ± 3.1
4	62.3 ± 3.8
0	59.4 ± 3.7
2	59.6 ± 2.9
2.5	53.9 ± 4.7
3	52.5 ± 3.2
3.5	56.3 ± 3.4

[a] Values are mean ± SE (N = 10 caudae); concentration was approximately 0.5 mg epididymal fluid/ml.

Sperm motility values in animals treated with dinoseb or α-chlorohydrin are given in Table 4. The 2 lowest dietary concentrations of dinoseb (125 and 150 ppm) produced a 40-45% decrease in the MI while 175 ppm reduced the MI to near zero. The lowest dosage of α-chlorohydrin (325 mg/kg) reduced motile spermatozoa by 45% and the highest dosage (475 mg/kg) reduced the MI by 70%. Many spermatozoa in the α-chlorohydrin-treated animals were twitching but lacked progressive movement. The spermatozoa concentration in the epididymal fluid was not affected by either compound.

The estimation of sperm motility in rats treated with dinoseb presented problems not encountered in control animals. At the highest dosage, decapitate and degenerating spermatozoa and *in situ* clumping of dead cells were observed. Dispersion of the clumped cells was inadequate and representative measurements of nonmotile and motile spermatozoa were not possible in many samples. Moreover, electronic counts of samples containing significant numbers of fragmented spermtails were unreliable. In the presence of severe effects on the reproductive tract, quantitative measurements of sperm motility were of limited value.

Table 4. Sperm Motility in Rats Treated with Dinoseb or α-Chlorohydrin

Dinoseb			α-Chlorohydrin		
Dietary Level ppm	N[b]	Motility Index	Dosage mg/kg	N	Motility Index
0	19	61.8 ± 1.9	0	19	56.5 ± 2.9
125	16	33.7 ± 4.0[c]	325	20	31.2 ± 2.8[c]
150	17	38.4 ± 4.1[c]	400	19	27.2 ± 3.3[c]
175	9	4.4 ± 4.7[c]	475	18	16.7 ± 4.3[c]

[a] Values are mean ± SE
[b] N = caudae
[c] Differs from control ($p < 0.05$)

Sperm motility assays are potentially useful adjuncts to several basic toxicity tests. In the present study we have provided the details of a simple objective procedure to estimate the proportion of motile rat spermatozoa under specified conditions. Further, we have demonstrated the reproducibility and applicability of the measurement in the test situation. The techniques described for sample collection and handling may also be useful with other quantitation techniques and for evaluating other parameters of sperm motility in the rat.

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